Perfluoroalkylated acids (PFAAs) accumulate in field-exposed snails (Cepaea sp.) and affect their oxidative status

Perfluoroalkylated acids (PFAAs) accumulate in field-exposed snails (*Cepaea sp.)* and affect their oxidative status

Jet Rijnders[a,], Lieven Bervoets[a], Els Prinsen[c], Marcel Eens[b], Gerrit T.S. Beemster[c], Hamada AbdElgawad[c,d] , Thimo Groffen[a,b]

[a]Systemic Physiological and Ecotoxicologal Research (SPHERE), Department of Biology, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium
Lieven.Bervoets@uantwerpen.be
Thimo.Groffen@uantwerpen.be
Jet.Rijnders@uantwerpen.be
[b]Behavioural Ecology and Ecophysiology Group (BECO), Department of Biology, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium
Marcel.Eens@uantwerpen.be
[c]Integrated Molecular Plant Physiology Research (IMPRES), Department of Biology, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium
[d]Botany and Microbiology Department, Faculty of Science, Beni–Suef University, Beni–Suef, 62521, Egypt
Els.Prinsen@uantwerpen.be
Hamada.Abdelgawad@uantwerpen.be
Gerrit.Beemster@uantwerpen.be

Abstract

Perfluoroalkyl acids (PFAAs) are a group of synthetic persistent chemicals with distinctive properties, such as a high thermal and chemical stability, that make them suitable for a wide range of applications. They have been produced since the 1950s, resulting in a global contamination of the environment and wildlife. They are resistant to biodegradation and have the tendency to bio-accumulate in organisms and bio-magnify in the food chain. However, little is known about the bioaccumulation of PFAAs in terrestrial invertebrates, including how they affect the physiology and particularly oxidative status. Therefore, we studied the bioaccumulation of PFAAs in snails that were exposed for 3 and 6 weeks along a distance gradient radiating from a well-known fluorochemical hotspot (3M). In addition, we examined the potential effects of PFAAs on the oxidative status of these snails. Finally, we tested for relationships between the concentrations of PFAAs in snails with those in soil and nettles they were feeding on and the influence of soil physicochemical properties on these relationships. Our results showed higher concentrations of PFOA and/or PFOS in almost every matrix at the 3M site, but no concentration gradient along the distance gradient. The PFOS concentrations in snails were related to those in the nettles and soil, and were affected by multiple soil properties. For PFOA, we observed no relationships between soil and biota concentrations. Short-chained PFAAs were dominant in nettles, whereas in soil and snails long-chained PFAAs were dominant. We found a significant positive correlation between peroxidase, catalase and peroxiredoxins and PFAA concentrations, suggesting that snails, in terms of oxidative stress (OS) response, are possibly susceptible to PFAAs pollution.

Keywords: Terrestrial ecosystem; Invertebrate; Soil; Plant; Oxidative stress; Antioxidants

Capsule: We observed a positive correlation between the levels of PFAAs and the antioxidants peroxidase, catalase and peroxiredoxins in snails, exposed on nettles grown at contaminated sites.

1. Introduction

Since 1950 the use of perfluoroalkylated acids (PFAAs) has gained vast interest from the chemical industry because of their distinct chemical properties. They are thermally and chemically stable and resistant to biodegradation (Li et al., 2018). Due to their amphiphilic character (Zhao et al., 2013b), PFAAs are widely used as e.g. surfactants, in pesticides, coatings, fast-food packaging and firefighting foams. As a result of their production and use, PFAAs have been distributed globally in the environment and wildlife (e.g. Giesy and Kannan, 2001; Kannan, 2011; Zhao et al., 2016). They can enter the environment via point sources, such as industry, and via indirect or diffuse sources, such as agricultural land where recycled wastewater was used for irrigation (Li et al., 2018). Due to their resistance to biodegradation, PFAAs tend to bio-accumulate. They also have the tendency to bio-magnify, leading to higher concentrations in organisms at higher trophic levels (Boisvert et al., 2019). Their potential to accumulate increases with the number of fluorinated carbon atoms (Oeritz et al., 2013; Wang et al., 2017). Although the bioaccumulation has been studied in terrestrial organisms at higher trophic levels (D'Hollander et al., 2014; Wang et al., 2017; Zhang et al., 2019), only little is known on invertebrates.

Soils are known to play a key role in the bioavailability of PFAAs to biota that form the basis of the terrestrial food chain. Human exposure to PFAAs has been linked to PFAS uptake by crops grown on contaminated soils before (Liu et al., 2019). Soil pollutants may retain in the soil for long periods due to their high persistency and may even spread to a large area due to leaching to deeper soil layers or the ground water (Simcik and Dorweiler, 2005; Xiao et al., 2015). Due to the amphiphilic properties of PFAS, their environmental fate and behaviour is governed by both electrostatic and hydrophobic interactions (Higgins and Luthy, 2007). Hence, their sorption to soils is influenced by numerous soil properties, such as organic carbon content, clay content, and temperature (Milinovic et al., 2015; Campos Pereira et al., 2018; Groffen et al., 2019d), and there are still uncertainties on how these properties interact to determine the binding of PFAAs to soils (Li et al., 2018) and the subsequent uptake by biota (Wen et al., 2014; Bizkarguenaga et al., 2016; Zhao et al., 2016).

Besides the limited knowledge on bioaccumulation and –magnification at the basis of the terrestrial food chain, data on toxicity of PFAAs to terrestrial invertebrates is also scarce (Zhao et al., 2013a). Understanding the toxicity to invertebrates is crucial, because toxic effects may affect the entire food chain. Frequently used measures of toxicity are physiological responses, such as the induction of antioxidant enzymes, which are an indication of the production of reactive oxygen species (ROS) and are involved in the detoxification of these ROS (Wen et al., 2011). Oxidative stress (OS) is the disturbance in the balance between the production of ROS and the antioxidant defenses of an organism.

Most studies that examined the effect of PFAAs on oxidative status in terrestrial invertebrates were conducted under laboratory conditions, targeted *Eisenia fetida,* and only included a small number of PFAAs (mainly PFOS and PFOA; Xu et al., 2013; Zhao et al., 2017), whereas data on other invertebrate taxa is mostly lacking.

According to Xu et al. (2013), malondialdehyde (MDA) content, a product of fatty acid peroxidation, rose significantly in earthworms with increasing duration of PFOS exposure, indicating that exposure to PFOS resulted in increased ROS levels. This increase in ROS correlated with increased antioxidant levels: superoxide dismutase (SOD), peroxidase (POX), catalase (CAT) and glutathione peroxidase (GPX) activities first increased and then decreased. Earthworms exposed to PFOA also had increased MDA levels and activities of SOD, CAT, POX, and glutathione S-transferase (GST). However, with increased exposure duration, the activities of these antioxidant enzymes decreased (Zhao et al., 2017).

However, these studies only examined a small number of oxidation products (mainly MDA), or enzymes involved in the conversion of ROS to non-toxic substances (mainly SOD, GPX, GST, POX, and CAT), whereas other enzymes involved in these processes have not been related with exposure to PFAAs before.

In this study, we investigated the bioaccumulation of PFAAs in snails, exposed to nettles and soil contaminated with PFAAs, along a distance gradient starting at a fluorochemical plant. In addition, we examined the relationship between the accumulated PFAAs concentrations and the oxidative status

of the snails. Finally, we investigated the correlations between PFAAs concentrations in the soil, nettles and snails and the influence of multiple soil physicochemical properties (organic carbon content, clay content, soil temperature, and pH).

Previous studies near this fluorochemical plant reported decreasing PFAAs concentrations with increasing distance from the 3M company in isopods (Groffen et al., 2019a,), in great tit (*Parus major*) (Groffen et al. 2017, 2019b; Lopez-Antia et al., 2019) and in wood mouse (*Apodemus sylvaticus*) (D'Hollander et al. 2014). Therefore, we expected to see the same trend of PFAAs concentrations in soil, nettles and snails along this distance gradient. Furthermore, we expected that the antioxidant system will become more active from a certain threshold level of accumulated concentrations of PFAAs and that concentrations of antioxidant metabolites and activity of enzymes that scavenge ROS will increase.

2. Materials and methods

2.1 Study area, sample collection and experiment setup

We used the snail *Cepaea sp.* (F. Helicidae, O. Stylommatophora, Cl. Gastropoda) as model species because snails have proven to be suitable sentinel indicators due to their wide distribution, easy sampling and their ability to accumulate various pollutants (Beeby and Richmond, 2002; Al-Alam et al., 2019). In addition, snails integrate different sources and paths of contamination as they live at the soil-plant-air interface (De Vaufleury et al., 2006; Pauget et al., 2013).

Around 520 individuals (weight of the snails is given in Table S1) were collected on September 4th, 2018 at a location with no history of contamination with PFAAs in Ghent (Eke, nearby the Grenadierslaan), Belgium. After spending a night in a climate chamber at 15 °C, the snails were exposed in cages at five different locations (Fig. 1). Based on prior monitoring studies (e.g. Dauwe et al., 2007; Groffen et al., 2019a, 2019b; Lopez-Antia et al., 2019), we selected the 3M fluorochemical plant in Antwerp, Belgium, Vlietbos (VB;1 km SE from 3 M), Middenvijver-Rot (MV; 2.3 km ESE from 3M) and Burchtse Weel (BW; 3 km SE from 3M) as study sites. As a reference site, we selected Westmalle (25 km NE from 3M).

At each location, we placed five cylindric stainless-steel cages (1 mm thick, 25 cm diameter, 25 cm height) over nettles (*Urtica dioica*). We placed 20 snails in each cage, randomly taken from the snails collected in Ghent and closed the cages with woven wire mesh. After three weeks, we randomly collected 10 snails per location and weighed them (Mettler AT261 DeltaRange, 0.001 g). In some cases, fewer than 20 snails were retrieved. However, we observed no mortality, as empty shells were not found inside the cages, suggesting that some snails must have escaped the cages during the study. If less than 10 snails were present, we collected all snails. Three weeks later, we collected the other 10 snails and treated them similarly. The selected exposure duration was based on previous studies using caged organisms to assess pollutant bioaccumulation (e.g. Gust et al., 2014; Regoli et al., 2006) as well as on laboratory studies examining PFAAs accumulation in terrestrial invertebrates (e.g. Xu et al., 2013; Rich et al., 2015). The majority of these studies report exposure durations of 4-6 weeks. Furthermore, this duration was similar to recent studies examining PFAAs bioaccumulation in caged invertebrates in the aquatic environment (Babut et al., 2020; Teunen et al., 2021). At 3M, there were only five snails left after six weeks of exposure, so these were used for the analysis of PFAAs only. Therefore, no results are present for oxidative stress parameters at 3M after six weeks. From each location, we stored half the number of snails at -20°C for analysis of PFAAs or at -80°C for OS analysis. After six weeks, we collected leaves and stems of the nettles, on which the snails fed in each cage, and pooled them per cage in polypropylene (PP) tubes at -20°C. Finally, at each site, we collected five soil samples (surface layer; 0 – 5 cm depth; Groffen et al., 2019d) using a stainless-steel shovel. We measured the pH and temperature of the soil, using a portable multimeter (HI9125, Hanna Instruments). The samples were stored in 50 mL PP tubes and stored at -20°C until further analyses.

2.2 Sample extraction and analysis

Four perfluorosulfonic acids (PFSAs, i.e. C4, C6, C8 and C10) and eleven perfluorocarboxylic acids (PFCAs, i.e. C4 – C14) were used as target analytes (Table S2). Isotopically mass-labeled internal standards (ISTDs) were purchased from Wellington Laboratories (Guelph, Canada) and contained

145 labeled standards of PFSAs (C6 and C8) and PFCAs (C4, C6, C8, C9, C10, C11 and C12) (Table S2). All
146 used abbreviations for the PFAAs analytes are according to Buck et al. (2011). .
147 Prior to analyses, we removed the shells of the snails, and homogenized soft tissues using a TissueLyser
148 LT (Qiagen GmbH, Germany) with stainless steel beads (5 mm; Qiagen GmbH, Germany). We
149 homogenized the nettles with a mortar and pestle using liquid nitrogen and oven-dried the soil
150 samples at 60°C.
151 The extraction procedure of snails and nettles was based on the method described by Powley et al.
152 (2005) with minor modifications. To each plant (± 0.25 g dry weight (dw)) or snail (± 0.75 g wet weight
153 (ww)) sample, we added 10 ng of each ISTD and 10 mL of acetonitrile (ACN; LiChrosolv, Merck
154 Chemicals, Belgium), after which we vortexed the samples, sonicated them for 3 x 10 min (Branson
155 2510) with vortexing in between pulses, and left them overnight on a shaking plate (135 rpm, room
156 temperature, GFL 3020, VWR International, Leuven, Belgium). After centrifugation (2400 rpm (1037 x
157 g), 4°C, 10 min, Eppendorf centrifuge 5804R, rotor A-4-44), we transferred the supernatant to a new
158 PP tube and concentrated it under vacuum to a remaining volume of approximately 0.5 mL using a
159 rotational-vacuum concentrator (Martin Christ, RVC-2-25, Osterode am Harz, Germany).
160 Subsequently, we transferred the extracts to an Eppendorf tube, containing 0.1 mL of graphitized
161 carbon powder (Supelclean ENVI-Carb, Sigma-Aldrich, Belgium) and 50 μL of glacial acetic acid. We
162 rinsed the PP tubes twice with 250 μL of ACN, which was pooled with the sample. After vortexing the
163 samples for at least 1 min and centrifugation (10.000 rpm (9279 x g) at 4°C for 10 min, in an Eppendorf
164 centrifuge 5415R), we transferred the liquid phase of the extracts to new Eppendorf tubes,
165 concentrated it until near dry, and reconstituted it in 200 μL of 2% ammonium hydroxide (HPLC grade;
166 Filter Service N.V., Belgium) diluted in ACN.
167 The extraction of oven-dried soil samples (± 0.30 g dw) followed the protocol described by Groffen et
168 al. (2019c). The protocol follows the same steps as described previously for biota, until the first
169 centrifugation step (4°C, 2400 rpm (1037 g), 10 min, Eppendorf centrifuge 5804R, rotor A-4-44).
170 Thereafter, we loaded the samples on Chromabond HR-XAW Solid Phase Extraction (SPE) cartridges (3

mL, adsorbent weight 200 mg, Macherey-Nagel, Germany), which were preconditioned and equilibrated with 5 mL of ACN and 5 mL of Milli-Q water (MQ; 18.2 mΩ; TOC: 2.0 ppb; Merck Millipore, Belgium), respectively. Hereafter, we washed the cartridges with 5 mL of 25 mM ammonium acetate (VWR International, Belgium) in MQ and 2 mL of ACN and eluted them with 2 x 1 mL of 2% ammonium hydroxide in ACN. We dried the eluent using a rotational-vacuum concentrator (Martin Christ, TVC-2-25, Osterode am Harz, Germany) and reconstituted it with 200 µl of 2% ammonium hydroxide in ACN. Finally, we vortexed all samples for at least 1 min and filtered them through Ion Chromatography Acrodisc 13 mm Syringe Filters with 0.2 mm Supor (polyethersulfone (PES)) Membrane (VWR International, Leuven, Belgium) attached to PP auto-injector vials.

2.3 UPLC-MS/MS analysis and quantification

After extraction, we analyzed the PFAAs using Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS, ACQUITY, TQD, Waters, Milford, MA, USA), with electrospray interface operating in negative ion mode (ES(-)-MS/MS). An ACQUITY BEH C18 column (2.1 × 50 mm; 1.7 µm, Waters, USA) separated the target analytes. An ACQUITY BEH C18 pre-column (2.1 × 30 mm; 1.7 µm, Waters, USA) was inserted between the solvent mixer and the injector to retain any PFAAs contamination originating from the system. The mobile phase solvents were A) 0.1 % formic acid in water and B) 0.1 % formic acid in ACN. The flow rate was set at 450 µl min^{-1} with an injection volume of 10 µl. The gradient started at 65 % A/35% B, decreased in 3.4 min to 0 % A/90% B and returned to 65% A/35% B at 4.7 min. The targeted PFAAs were identified and quantified by multiple reaction monitoring (MRM) using two diagnostic transitions per target analyte. The diagnostic transitions used are listed in Table S2.

2.4 Oxidative stress and antioxidant analysis

We freeze dried, weighed and homogenized five snails per location and per sampling time. We determined lipid peroxidation by monitoring the production of malondialdehyde (MDA) according to Hodges et al. (1999), the total antioxidant capacity using the ferric reducing/antioxidant power (FRAP) assay, described by Benzie and Strain (1999), the total polyphenolic content by the Folin Ciocalteu

assay (Zhang et al., 2006), using gallic acid as a standard, and the total flavonoid content, using the modified aluminum chloride colorimetric method (Chang et al., 2002), using quercetin as standard. We prepared the protein extracts for the antioxidant enzymatic assays according to Murshed et al. (2008) and quantified them according to Lowry et al. (1951). We determined the enzyme kinetics at 25 °C on 20 µl of protein extract using a micro-plate reader. We measured the activity of superoxide dismutase (SOD) according to the procedure of Dhindsa et al. (1982), catalase (CAT) according to Aebi (1984), glutathione peroxidase (GPX) according to Drotar et al. (1985) and xanthine oxidase (XO) according to the method of El-Soud et al (2013). We assessed POX according to the method of Kumar and Khan (1983), and spectrophotometrically determined the activity of glutathione-S-transferase (GST), glutathione reductase (GR), monodehydroascorbic acid (MDHAR), dehydroascorbic acid reductase (DHAR) and ascorbic acid peroxidase (APX) based on the method of Murshed et al. (2008). Finally, we determined the peroxiredoxin (PRX) activity according to Horling et al. (2003), Glutaredoxin (GRX) activity according to Lundberg et al. (2001) and thioredoxin (TRX) activity according to Wolosiuk et al. (1979).

2.5 Soil characteristics

We determined the soil moisture content using the weight loss between wet (10 g) and oven-dried (70°C, 48h) soils. We used a Malvern Mastersizer 2000 and Hydro 2000G to determine the clay content, after sample (1 g fresh soil) pre-treatment with 40 mL of 33% hydrogen peroxide and 9 mL of 30% hydrochloric acid to destruct iron conglomerates and organic materials in the samples. Finally, we determined the total organic carbon (TOC) content using the loss on ignition (LOI) method described by Heiri et al. (2001). Briefly, oven-dried (60°C) soil samples (2 g) were dried in a muffle furnace at 105°C for at least 24h and weighed. Hereafter, the samples were incinerated in the muffle furnace at 550°C for at least 5h and the weight loss was determined. We calculated the TOC using Formula 1.

$$TOC\ (\%) = 100 * ((DW_{105} - DW_{550}) / DW_{105}) / 1.742 \quad (1)$$

With DW_x the dry weight of the sample after heating at 105°C or 550°C, respectively. The "Van Bemmelen factor" of 1.742 was applied since it is assumed that 58 % of the total organic matter is carbon (Nelson and Sommers 1996).

2.6 Quality assurance

In order to limit the variation in mass and size between different individuals and hence the potential influence of these factors on PFAA concentrations, we randomly placed the snails at the different locations. The weight of the snails (Table S1) did not differ among the different sites ($F_{4,237}$=0.57, p=0.681).

For the PFAA analysis, we used procedural blanks (10 ml of ACN) for quality control at a ratio of one procedural blank per 20 samples. The concentrations in the blanks were all below the quantification limit (<LOQ). The recoveries of the samples varied between 1% and 100%. Recovery losses for each compound of interest in the individual samples were taken into account using the internal standard. Samples with recoveries <10% were not included in further analyses. The individual LOQs were calculated based on a signal-to-noise (S/N) ratio of 10 normalized with the compound and sample specific recoveries (Table 1).

2.6 Bioconcentration and accumulation

The bioconcentration factor (BCF) from soil to nettles and snails was calculated as the ratio between the concentration of the different PFAAs compounds in biota and the concentration in the soil. The bioaccumulation factor (BAF) from nettles to snails was calculated as the ratio between the concentration of the different PFAAs compounds in snails and nettles. Both factors could only be calculated when the compound was measured in all of the matrices involved for the factor.

2.7 Statistical analyses

We performed the statistical analyses in R (version 3.2.3). The level of significance for all the tests was set at $p \leq 0.05$. Whenever concentrations of a given analyte were below the LOQ in more than 50% of the samples, the compound was excluded from analyses. Concentrations that were below the LOQ were given a concentration of LOQ/2 (Bervoets et al., 2004; Groffen et al., 2017; Lasters et al., 2019),

in order to minimize left-skewness of the data. The Shapiro-Wilks test was used to test the data for normal distribution. A log-transformation was used whenever these assumptions were not met. We expected a decrease in PFAA concentrations with an increased distance from the 3M chemical plant, and accordingly changes in antioxidant activity. Therefore, a two-way ANOVA, followed by a Tukey post-hoc analysis, was used to investigate significant differences in PFAA concentrations and antioxidant parameters among locations and between time periods (3 weeks or 6 weeks). As we expected the antioxidant system to become more active from a certain threshold of accumulated PFAAs, we also tested the correlation between the PFAAs concentrations and the levels of antioxidants. First, a principal component analysis was conducted on the detected PFAAs in order to account for collinearity among the different PFAAs. Two principal components were selected, which together explained almost 90% of the total variation in the dataset. The loadings of both PCs can be found in Table S3. The first PC (PC1), which is influenced by PFBA, PFOA, PFDA, PFUnDA, PFDoDA and PFOS, explained 68% of the variation. Low values of these compounds correspond with a high value of PC1. The second PC (PC2) explained almost 20% of the variation and was influenced by PFBA, PFNA, PFDA, PFUnDA and PFOS. High PC2 values indicate high PFBA and PFOS concentrations, but low PFNA, PFDA and PFUnDA concentrations (Table S3). Hereafter, we used multiple linear regression to relate the PFAA concentrations to the antioxidant concentrations. As PFAAs tend to bioaccumulate in the food chain, we expected the PFAAs concentrations in the soil to be correlated with those in nettles, and we expected a similar correlation between the PFAA concentrations in nettles and snails. In these analyses, we converted the PFAA concentrations in the snails (as reported in Table 1 and Tables S6 and S7) to ng g^{-1} dw, based on an average (±St. Dev.) moisture content of 79.6 ± 1.5 %, as determined in five snails from Ghent. A multiple linear regression was used when investigating the influences of soil parameters on the relationships between PFAA concentrations in the different matrices (soil, snails and nettles) and on the BCF of the PFAA concentrations in nettles. The Akaike Information Criterion (AIC) was used to select the best fitting model. For the correlation between PFAA concentration in snails and nettles, a spearman rank correlation test was used.

3. Results

3.1 PFAA concentrations in the soil's surface layer

To investigate the spatial distribution of PFAAs in the environment, as well as the bioaccumulation from soil to nettles and snails (section 3.4), we examined the PFAA concentrations in the soil surface layer along the distance gradient from the 3M fluorochemical plant.

In the soil's surface layer, PFBA, PFOA, PFUnDA and PFOS were detected in more than 50% of the samples from at least one sampling site (Table 1 for mean values, Table S4 for more details), whereas PFPeA, PFHxA, PFHpA, PFNA, PFDA, PFDoDA, PFTrDA, PFTeDA, PFBS, PFHxS and PFDS were not detected in any of the samples. These latter compounds were therefore omitted from the statistical analyses as is motivated in section 2.7.

Concentrations of PFUnDA could not be compared among sites, as it was only detected at Vlietbos, suggesting a potential local, but unknown, source of PFUnDA at this site. Significant differences among sites were observed for PFBA and PFOS, but not for PFOA. The PFBA concentrations in the surface layer at Middenvijver-Rot were significantly higher than those detected at VB ($p < 0.001$) and BW ($p = 0.003$) and the PFOS concentrations were higher at Middenvijver-Rot compared to 3M ($p = 0.014$) and Burchtse Weel ($p < 0.001$).

3.2 PFAA concentrations in nettles

Concentrations of PFPeA, PFHpA, PFTrDA, PFTeDA, PFHxS and PFDS in nettles were all below the limit of quantification (LOQ). PFBA, PFOA, PFDA and PFUnDA were detected at all locations, whereas PFOS was only detected at 3M, Middenvijver-Rot and Burchtse weel, PFHxA only at Vlietbos and Westmalle, PFNA only at Westmalle, and PFBS only at 3M (Table 1 for mean values, Table S5 for more details).

Concentrations of PFBA in nettles differed significantly among sites ($p<0.001$, $F_{3,16}=71.4$), with significantly higher concentrations at 3M compared to Burchtse Weel ($p<0.001$), Middenvijver-Rot ($p=0.001$), and Vlietbos ($p<0.001$). PFOA concentrations also tended to be higher at 3M than at Middenvijver-Rot ($p = 0.094$, $t_8 = -1.90$). PFDA concentrations did not differ between Vlietbos and

298 Burchtse Weel ($p = 0.152$, $t_8 = 1.58$) and concentrations of PFUnDA did not differ significantly among
299 the four locations ($p = 0.506$, $F_{3,16} = 0.812$).

300 3.3 PFAA concentrations in snails

301 After three (Table 1 for mean values, Table S6 for more details) and six (Table 1 for mean values, Table
302 S7 for more details) weeks of exposure, PFBA, PFOA, PFNA, PFDA, PFUnDA, PFDoDA and PFOS were
303 detected in the snails. With exception of Ghent (i.e. location where the snails were collected; Fig. 1),
304 where PFHxA was detected at a concentration of 0.499 ng g^{-1} ww in one snail, PFPeA, PFHxA, PFHpA,
305 PFTrDA, PFTeDA, PFBS, PFHxS and PFDS were not detected in any of the snails.
306 When all the measurements of the two time periods were combined per site, only PFUnDA was
307 detected in more than 50% of the samples in Westmalle and Middenvijver-Rot, but the concentration
308 did not differ significantly between both locations ($p=0.506$, $t_9=-0.69$). PFOA and PFOS were only
309 detected in more than 50% of the samples at Middenvijver-Rot, while PFNA, PFDA and PFDoDA were
310 not detected in more than 50% of the samples at any location. PFBA on the other hand was only
311 detected at 3M.

312 3.4 Relationships between the soil and biota concentrations and influence of soil characteristics

313 In order to investigate the bioavailability and bioaccumulation from soil to nettles and snails, we
314 assessed the relationships between the soil and biota concentrations. In addition, we examined the
315 influence of soil characteristics (TOC, clay content, soil temperature, moisture content and pH; Table
316 S8) on these relationships, and hence on the bioavailability from soil to biota. Because only PFOS and
317 PFOA were present in more than 50% of the snail and soil samples, we focused our analysis on these
318 compounds. All p- and R^2-values of the significant relationships are shown in Table 3.
319 The PFOS concentrations in nettles were indeed significantly positively related to PFOS concentration
320 in the soil ($p<0.001$; Table 3). In contrast, no correlation between PFOA concentrations in soil and
321 nettles was found ($p>0.05$, Table 3).
322 The uptake of PFOS from soil by nettles was negatively influenced by the soil temperature (log-lin,
323 $p=0.001$; Table 3) and moisture content (log-lin, $p=0.007$, Table 3). Similar to the nettles, PFOA

concentrations in the snails were also unrelated to those in the soil (log-lin, p=0.513, Table 3), whereas the PFOS concentrations in the snails were negatively related to the soil concentrations (lin-lin, p=0.004, Table 3). The bioavailability of PFOS from soil to the snails was positively affected by the soil pH (lin-lin, p=0.004, Table 3) and temperature (lin-lin, p=0.006, Table 3) and negatively by TOC (lin-lin, p<0.001, Table 3) and moisture content (lin-lin, p=0.009, Table 3).

Finally, for both PFOA (lin-log, p=0.004, df= 22, R^2 = 0.561) and PFOS concentration (p=0.04, df= 22, R^2 = 0.426), a significant positive correlation was found between snails and nettles (lin-log, Table 3).

The BCF of nettles (Table 4) for PFBA was positively influenced by pH (lin-lin, p<0.001, R^2=0.004) and temperature (lin-lin, p<0.001 and R^2 0.115) and negatively influenced by moisture content (p<0.001, R^2 = 0.042) whereas no significant influence was found for TOC (lin-lin, p=0.94, R^2 = 0.024) or clay (lin-lin, p=0.296, R^2 = 0.004). Although the BCF of nettles for both PFUnDA and PFOA was not significantly influenced by clay (lin-lin, p=0.322, R^2= 0.001 for PFUnDA and lin-log, p = 0.153, R^2 <0.001 for PFOA), it was negatively influenced by pH (lin-lin, p<0.001, R^2< 0.001 for PFUnDA and lin-log, p<0.001, R^2 < 0.001 for PFOA), TOC (lin-lin, p<0.001, R^2 = 0.021 for PFUnDA and lin-log, p<0.001, R^2 <0.001 for PFOA), moisture content (lin-lin, p<0.001, R^2 = 0.188 for PFUnDA and lin-log, p <0.001, R^2 = 0.082 for PFOA) and temperature (lin-lin, p=0.022, R^2 = 0.010 for PFUnDA, lin-log, p = 0.006, R^2 = 0.001 for PFOA).

3.5 Oxidative damage and antioxidant parameters

Next, we set out to investigate the impact of PFAAs on oxidative stress and antioxidant status of the snails (Fig. 2, Table S9).

To identify functional relationships, we performed Principle components analyses using the levels of PFAAs to explain variation in antioxidant activities. None of the oxidative stress parameters showed a relationship with PC1 (all p ≥ 0.06), which reflects low values of PFBA, PFOA, PFUnDA, PFDoDA and PFOS (Table S3). The second principal component, PC2, indicates high PFBA and PFOS concentrations, but low PFNA, PFDA and PFUnDA concentrations, and showed a significant positive relationship with POX (lin-lin, p= 0.01, $F_{(1, 0.03)}$ = 11.38), CAT (lin-lin, p = 0.04, $F_{(1, 4.73)}$ = 6.78), GPX (lin-lin, p= 0.02, $F_{(1, 2.3*10^{-}}$

$_{4)}$ = 8.46) and peroxiredoxins (lin-lin, $p < 0.01$, $F_{(1, 3.7*10^{-5})} = 14.15$) (Fig. 3). However, no relationship between PC2 and MDA or other antioxidant enzyme activities was found (all P≥0.09).

4. Discussion

4.1 PFAA concentrations in the soil's surface layer

With exception of PFBA, only long-chained PFAAs (i.e. PFOA, PFOS and PFUnDA) were detected in the soil's surface layer. This was expected, because soil concentrations depend to some extent on the carbon-chain length. The sorption rate for the longer-chained compounds is higher due to their higher hydrophobicity (Ahrens et al., 2010; Higgins and Luthy, 2006).

The concentrations of most PFAAs were higher in surface soils collected at 3M, Vlietbos, Middenvijver-Rot and Burchtse Weel in 2016 (Groffen et al., 2019a). However, the PFBA and PFOS concentrations at Middenvijver-Rot were higher in the present study, likely due to differences in soil physicochemical properties (e.g. TOC) or sampling strategy (Groffen et al., 2019d). These concentrations are within the range of those reported in other international studies. The PFBA concentrations in surface soil near a fluorochemical park in China (0.6 ng g^{-1} dw, Lu et al., 2018) were similar to those observed at Vlietbos, but lower than those observed at Middenvijver-Rot and Burchtse Weel. The PFOA (50.1 ng g^{-1} dw) and PFOS (2583 ng g^{-1} dw) concentrations at a fluorochemical manufacturing facility in Wuhan, China were higher than those reported in the present study at 3M, whereas the concentrations of PFOA (0.79 ng g^{-1} dw) and PFOS (7.06 ng g^{-1} dw) near a fluorochemical plant in Hubei Province, China were similar (Wang et al., 2010). However, it is often unclear in these studies to which depth soil samples were collected, and hence it is possible that these comparisons were based on different soil layers.

In another study from Groffen et al. (2019a), the PFAAs concentrations in the surface's soil progressively decreased with distance from the fluorochemical plant, which is in contrast to our results. The lack of such decrease in the current study could potentially be attributed to differences in sampling strategy and soil physicochemical properties compared to the previous study by Groffen et al. (2019a), resulting in lower concentrations in the surface soil collected at the plant site.

4.2 PFAA concentrations in nettles

As expected, nettles contained higher concentrations of the short-chained PFBA and PFBS compared to the longer-chained PFAAs. PFAAs uptake in plants is known to depend on the chain length (Krippner et al., 2015), as short-chained PFAAs have a higher adsorption into plant roots due to their smaller molecular size (Müller et al., 2016; Gredelj et al., 2020a). In addition, these short-chained analytes are more water-soluble and have a lower adsorption to soil (Ghisi et al., 2019; Gredelj et al., 2020a). The root affinity and shoot translocation of PFAAs is not solely predicted by hydrophobicity, but rather by a combination of both hydrophobicity and anion exchange (Blaine et al., 2013; Guelfo and Higgins, 2013). Casparian bands in the plant roots prevent apoplastic passage of ions from the cortex to the stele. Hence, any ion that is absent in the symplast will be prevented from entering the shoot system (Enstone et al., 2003). Long-chained PFAAs will be partially dissociated into their ions in an aqueous solution due to their lower pKa values (Moroi et al., 2001; Goss, 2008), and may consequentially enter the symplastic transport system of plants through which they end up in the leaves.

Despite the higher concentrations at the fluorochemical plant, our results do not provide evidence that concentrations decrease with increasing distance from the 3M site, as significant differences among locations were often lacking in the adjacent sites.

To the best of our knowledge PFAAs have only been studied in crop species (e.g. Blaine et al., 2013, 2014; Costello and Lee, 2020), and studies on native plants species are absent. As in these earlier studies plants were grown on spiked soils or biosolid-amended soils (e.g. Felizeter et al., 2012; Gredelj et al., 2020b), the exposure routes are different from our study and therefore we cannot compare our results with those obtained earlier.

4.3 PFAA concentrations in snails

As snails mainly feed on dead material and green fresh plants such as nettles (Williamson and Cameron, 1976), we expected some resemblance in PFAAs accumulation profiles between snails and nettles. However, except for PFBA and PFHxA, mainly long-chained PFAAs were traced in the exposed snails, compared to mainly short-chained PFAAs in the nettles (chapter 3.3). Hence, the PFAAs profiles in snails are more similar to those in the soil than those in nettles. Animals are known to mainly

401 accumulate long-chained PFAAs due to their lower water-solubility, higher affinity for proteins and
402 hence longer elimination half-lives compared to some short-chained homologues (Brendel et al.,
403 2018).
404 Field studies on PFAAs in terrestrial invertebrates are scarce. When we compare the concentrations in
405 snails in the present study with previous studies on terrestrial invertebrates near the Antwerp hot-
406 spot (D'Hollander et al., 2014; Groffen et al., 2019a), the concentrations in the present study are lower
407 at all sites, with exception of PFOS and PFOA at Middenvijver-Rot in the isopods reported by Groffen
408 et al. (2019a) (Table 2). To the best of our knowledge, besides the study of D'Hollander et al. (2014)
409 and Groffen et al. (2019a) only two other field studies were conducted on PFAAs in terrestrial
410 invertebrates. Median PFOS (highest median of 16 ng g^{-1} ww) and PFOA (highest median of 0.89 ng g^{-1}
411 ww) have been reported in adult Odonata from South Africa (Lesch et al., 2017). These concentrations
412 were much higher than those detected in the present study. Concentrations of PFDA and PFUnDA in
413 the Odonata species were, however, similar to those reported at the 3M site in the present study.
414 Finally, Zhu and Kannan (2019) reported PFAA concentrations in earthworms nearby a fluorochemical
415 manufacturing facility at the Little Hocking well field in Ohio, USA. The mean concentrations in this
416 study were all much higher (ranging between 1.2 ng g^{-1} dw for PFPeA to 270 ng g^{-1} dw for PFOA) than
417 those reported in snails in the present study (Table 2). The lower concentrations in the present study
418 are likely the result of a limited exposure time in the translocated snails in the present study compared
419 to the resident organisms that were collected in the other studies.
420 The absence of differences in PFAAs concentrations among locations also appears to indicate that our
421 study does not provide evidence for a pollution gradient in snails among these study sites. As the
422 detection frequency of the targeted analytes was below 50% both after three and after six weeks of
423 exposure, we were only able to investigate spatio-temporal differences in PFAA concentrations using
424 a dataset that was potentially left-skewed due to the amount of non-detects. Although the exposure
425 duration in the present study was similar to other laboratory studies and studies using translocated
426 organisms (as mentioned in chapter 2.1), and hence spatio-temporal differences were expected, future

research should take place over a longer time period with a larger number of replicates, to increase the exposure duration and reduce the chance of non-detects.

4.4 Relationships between the soil and biota concentrations and influence of soil characteristics

PFAAs accumulation in plants is known to depend on chain length and functional groups of the analytes (Ghisi et al., 2019). PFSAs generally accumulate to lesser extent than PFCAs in plants (Ghisi et al., 2019), which is contradictory to our findings.

Our results showed that the bioaccumulation of PFOS from soil to nettles was negatively influenced by temperature and moisture content. This appears to differ from previous findings in literature, where an increase in temperature induced higher transpiration rates and hence higher PFAA uptake in plants (Zhao et al., 2016). However, an increased temperature has been related to higher sorption of PFOS to soils (Groffen et al., 2019d; You et al., 2010), hence reducing bioavailability to plants. The role of soil moisture content on bioavailability of PFAAs to plants is, to the best of our knowledge, still unknown. However, soil moisture content is known to indirectly influence physical, chemical and biological soil properties, such as reduced solubility of nutrients caused by decreased redox potentials (Neumann and Römheld, 2012).

The PFOS uptake by snails from soil was negatively influenced by TOC and moisture content and positively affected by soil pH and temperature. TOC plays a key role in the sorption of PFAAs to soils (Campos Pereira et al., 2018) and higher TOC contents have been related to higher soil PFAAs concentrations and subsequently to lower PFAAs bioavailability (Groffen et al., 2019d; Wen et al., 2015; Zhao et al., 2016). As PFAAs are weakly acidic chemicals, an increased pH, increases the proportion of anionic molecules, resulting in a decreased sorption (Li et al., 2018), an increased bioavailability and higher PFOS concentrations in the snails. Furthermore, the net surface charge of mineral particles becomes less positive when pH increases (Johnson et al., 2007). As an increased temperature increases sorption and decreases bioavailability of PFOS (You et al., 2010), the positive effect of temperature on the bioavailability of PFOS from soil to snails was unexpected.

The BCF of nettles for PFOA was comparable to those found in a study by Knight et al. (2021), where the log BCF value for PFOA ranged from 0.1 - 1.6. Blaine et al. (2013) found comparable BCF values for PFOA (0.11 – 1.34) but our result for PFBA at 3M highly exceeded the BCF of PFBA found in that study (12-65). The BCFs of nettles for PFBA, PFOA and PFUnDA are higher at 3M compared to the other sites (Fig. 4). According to other studies (e.g. Lasee et al., 2020; Wen et al., 2014), TOC is an important factor for PFAA uptake. The lower the TOC, the better the uptake of PFAAs by plants. This is also confirmed in the present study for PFUnDA and PFOA, as the BCFs of nettles for these compounds were negatively influenced by TOC in the soil. As the TOC content at 3M was lower in the soil, this explains the higher concentration for PFUnDA and PFOA in nettles at 3M. However, for PFBA, this correlation was not found even though the shorter the PFAA chain, the bigger the influence of TOC on PFAA uptake by plants (Campos Pereira et al., 2018).

As the BCF of snails could only be calculated for PFUnDA at two locations, and for PFBA at only one location, no statistical analysis was performed to check for influences of soil characteristics on the BCF or the BAF, which could only be calculated for PFUnDA and PFBA both at only one location (Table 4).

4.5 Oxidative damage and antioxidant parameters

PC2 (reflecting high PFBA and PFOS concentrations, but low PFNA, PFDA and PFUnDA concentrations) was positively related to levels of POX, CAT, GPX and peroxiredoxins. These enzymes represent a first line of defense against reactive oxygen species (Bonato et al., 2020). Because we did not observe any relationship with MDA content, there was no increase in oxidative stress in the snails. Hence our results suggest that anti-oxidant defenses were sufficient to prevent oxidative damage caused by reactive oxygen species.

Other studies on earthworms also reported an effect of PFOS and PFOA on the activities of CAT and POX (Xu et al., 2013; Zhao et al., 2017). In both studies, the activities of CAT and POX were inhibited after 14d (Xu et al., 2013) or 18d (Zhao et al., 2017) of exposure. Nonetheless, both Xu et al. (2013) and Zhao et al. (2017) reported effects of PFOS and PFOA exposure on MDA and SOD levels, for which we did not observe any relationship.

However, it should be mentioned that these studies were carried out under laboratory conditions and focused on earthworms and species-specific differences in sensitivity to oxidative stress have been reported before in other invertebrate taxa (Walker et al., 2000). Furthermore, it should be noted that a translation of these results on OS should be interpreted with caution as other environmental factors and pollutants could affect the snail physiology. Therefore, exact mechanisms through which PFAAs induce oxidative stress effects should be investigated under controlled conditions as this allows exclusion of most confounding factors.

5. Conclusion

Although the PFAA concentrations in snails and nettles were higher at the fluorochemical plant compared to the other sites, we did not observe a decrease of PFAAs concentrations with increasing distance from 3M. Our results demonstrate bioaccumulation of PFOS in the food chain, from soil to nettles and snails, as well as from the nettles to the snails. Multiple soil properties affected these relationships, by influencing the transfer of PFOS from soil to nettles or snails. However, for PFOA we did not find bioaccumulation in this food chain. In addition, we did observe an association between the accumulated PFAA concentrations and the oxidative status in snails. High PFBA and PFOS concentrations, but low PFNA, PFDA and PFUnDA concentrations showed a significant positive relationship with POX, CAT, GPX, and peroxiredoxins. This suggests that snails are possibly susceptible to PFBA and PFOS pollution in terms of oxidative stress response. Therefore, continuous monitoring of the exposure and effects in invertebrates, using different invertebrate species, and taking into account different species sensitivities to PFAA pollution, is essential for a further understanding and for risk assessment of PFAAs in a terrestrial environment.

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